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(54) Title: MATRIX SEQUENTIAL ADDITION IMMUNOASSAY

(57) Abstract

Disclosed is a method for the rapid, quantitative assay of an analyte. The method utilizes flow through a matrix, e.g. a chromatography column, having attachment sites for a binding protein which is capable of specifically binding the sample analyte and an analog. The assay preferably is performed as a nonequilibrium-based sequential immunoassay. The analog does not need to be labelled to be identified, and the amount of free analog exiting the matrix is directly related to the amount of analyte bound to the matrix. The bond between the matrix attachment sites and the binding protein may be reversible, allowing the system to be regenerated *de novo* for each assay.

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MATRIX SEQUENTIAL ADDITION IMMUNOASSAY

Background of the Invention

The present invention is generally related to a method and apparatus for the assay of an analyte. More particularly, this invention relates to a rapid method for the quantitative assay of plural samples of analytes useful as a monitor of product quality during protein production.

The need to monitor product quality during protein production is well understood in the art. Process monitoring systems are of particular importance in the production of therapeutic substances for human use, where Federal regulatory agencies place stringent controls on product quality. To be useful, the monitoring system should be rapid, allowing the data generated to be used in a feedback system to adjust process parameters. The monitoring system should also be adaptable, easily altered to analyze different samples.

For the production of proteins produced by genetic engineering, product quality is a function of both purity and structure. Once expressed, the protein must be separated from all other cellular components, many of them present in excess of the protein of interest and/or having similar purification profiles.

For example, if the product is a therapeutic substance for human use, federal regulations require all contaminating components be removed to a level of less than 1 ng/dose of compound. This generally requires that the product be of at least 99.9% purity.

In addition, the native conformer must be separated from all variants of the protein which may arise as a result of the purification process or errors in biosynthesis. It is known that organisms which have been induced to express a protein can misincorporate amino acids at certain positions in the protein. In fact, up to 5% of the proteins produced can have such expression errors. Other biosynthetic errors also may be introduced during post-translational modification of the protein, including improper glycosylation, proteolysis, or incorrect disulfide bond formation, all of which can affect the protein's three dimensional structure.

Conformational changes to the protein are important because, in general, it is the native conformer of the protein that is biologically active. In addition to diminishing the biological activity of proteins, structural alterations in the protein may increase the protein's immunogenicity. This is particularly undesirable in therapeutic proteins that must be administered repeatedly.

Definitive methods of protein structure analysis such as X-ray crystallography, amino acid sequencing, or even peptide mapping are too time-consuming and labor-intensive to be used on a routine basis as methods of monitoring protein

quality. For analytical data on protein quality to be most useful in the manufacturing process, the data should be available to be used in a type of feedback system to allow adjustment of the process parameters. This requires that the time needed to determine protein quality be short relative to the processing time. Put another way, the analytical data should be generated in "real" time. If the quality assurance data is generated more slowly than the rate of production, it only allows one to discard or recycle unacceptable material.

High performance liquid chromatography (HPLC), high performance capillary electrophoresis (HPCE), rapid analytical immunoassay (RAIA), or some combination of these, are alternative analytical techniques that may be adapted for rapid process monitoring. Unfortunately, each of these techniques has limitations. Chromatography and electrophoresis analyze samples by separation, based on size or charge. During the early stages of purification the product may be contaminated with a large number of other proteins and constitute less than 10% of the total protein mass, making it difficult to identify the protein of interest by conventional separation techniques. Similarly, at the very late stages of protein purification, protein purity is very high, generally exceeding 95%. At high concentrations of a single product, a chromatogram or electropherogram is essentially a single peak, making it difficult to detect small concentrations of contaminants in this range. Separation methods here are also of limited utility.

In addition, chromatographic and electrophoretic analyses are limited by the time required to perform their protocols. The speed of HPLC analysis generally is limited by the porous chromatographic packings used in conventional HPLC chromatography. While mobile phase mass transfer between the matrix particles is convectional in HPLC chromatography, mobile phase mass transfer within the porous particles is primarily diffusional, substantially slowing the progress of the protein through the matrix. Similarly, the speed of electrophoresis separation is a function of the voltage applied across the system. However, heat production increases with the square of the voltage. Thus, the maximum voltage most electrophoresis systems will tolerate is limited by the speed with which they can dissipate the heat generated by the applied voltage.

Of the three alternative analytical techniques, rapid analytical immunoassay (RAIA) appears to be the only technique of sufficient discriminating power to isolate a protein of interest from both crude samples and substantially pure products. Moreover, because an immunoassay involves a specific binding interaction with the protein of interest, to a first approximation, the assay provides a method of determining if the protein is folded correctly. However, conventional analytical immunoassays, performed as equilibrium reactions, also are limited by the incubation times required in most assays.

Immunoassays are based on the well-known reactions between an antibody and the specific antigen or hapten (analyte) for that antibody, although the general principal can be applied to any specific binding interaction between a binding protein and its ligand. In general, an analog of the sample antigen or analyte competes with the sample antigen for complex formation with an antibody specific for that antigen. The competing analog is often a purified form of the sample antigen that is tagged, e.g., with a fluorescent or radioactive tracer material. The analog also can be tagged with an enzyme having a readily assayable activity, such as reacting with a substrate to give a colored product. The fraction of tagged analog bound tothe antibody (or remaining free in solution) is then measured, and is related to the amount of sample antigen (analyte) present in the reaction. It has generally proven easier to quantitate the bound species, as this species is often the easier of the two to collect.

Many approaches to the technique have been developed which differ mostly in the order and timing of the addition of sample antigen and tagged analog to the antibody, and in the method of separating bound and free species. (See Bolton et al., Handbook of Experimental Immunology, Weir, D.M., Ed., Blackwell Scientific Publications, Oxford, 1986; Vol. 1, chapter 26, for a general discussion on immunoassays.) Competition immunoassays, one of the most common methods used, involves the simultaneous

incubation of sample antigen and tagged analog with antibody until equilibrium is reached, separation of bound and free species, and the subsequent quantitation of one of these species. Because the sample antigen and the tagged analog compete for antibody binding sites, generally speaking, not all sample antigen is bound, reducing the sensitivity of the assay. A more sensitive assay is a sequential addition immunoassay (SAIA), also known as delayed addition assay, where sample antigen is added first and allowed to reach equilibrium with the antibody, followed by addition of tagged analog (or vice versa). SAIAs have been shown to be several times more sensitive than simultaneous incubation assays for a given concentration of sample antigen, as they allow all target antigen an opportunity to bind antibody before the addition of a competitor.

As stated earlier, quantitation generally requires separation of the bound and free components. Bound complexes can be collected by precipitation, usually with a second antibody (so called "sandwich" or "double antibody" assays.) Alternatively, one of the components (generally the antibody) can be immobilized on an easily separable solid support, such as a test tube wall or Sepharose beads. Immobilized immunoassays (called immunosorbent assays) comprising Sepharose beads are particularly attractive for analytical assays because the beads can be packed into a chromatography column, allowing the sample to be captured, and impurities to be washed away.

The antibody (or immobilized component) is generally covalently attached to the solid support. Alternatively, the antibody may interact reversibly with the solid support if the support's surface includes a component, such as protein A, capable of specific, reversible interaction with the antibody. If the immobilized component is reversibly bound to the solid support, the bound complexes can be eluted and the analog-antibody complexes quantitated. Quantitation requires that the analog be tagged in some way, to distinguish complexed analog from complexed sample antigen. See, for example, Mattiason et al., Proc. Int. Symp. on Enzyme-Labelled Immunoassay of Hormones and Drugs, Pal, S., Ed., Walter de Gruyter, Berlin (1978), p. 91.

If the immobilized component is covalently attached to the solid support, the sample antigen can be analyzed by simply eluting it from the column and quantitating it with a UV detector, potentially eliminating the need for a tagged competitor. Unfortunately, many compounds used to elute sample antigens, so-called eluting agents, coelute with the antigen and can cause a significant perturbation of the detection base line, making it difficult to differentiate the elution profile of the sample antigen from that of the eluting agent. In practice, this effect can diminish the sensitivity of the assay by more than 100 fold, depending on the detection wavelength and eluting agent used. A second disadvantage with this system, and of particular

importance for a rapid monitoring system, is that it requires covalent immobilization of the antibody or binding protein to prevent its elution with the antigen. This means that the column must either be dedicated to the analysis of a single antigen for its lifetime, or that the column be repacked each time a different sample antigen is to be sampled.

An additional concern with conventional analytical immunoassays as a monitoring protocol is that the requirement for equilibrium conditions often demands substantial incubation times. In fact, U.S. Patents Nos. 4,816,418 and 4,128,628 each describe an apparatus for the automated assay of multiple samples in sequence that attempt to overcome the limitation imposed by reaction incubation times. In each apparatus the samples can be sidetracked to incubate and are retrieved to quantify after the reactions have reached equilibrium. A typical assay may take ten or more minutes to perform, which is often too long for feedback analysis.

As used herein, the following terms have the following meanings:

"Analyte", as used herein, includes any sample compound to be analyzed that is capable of being bound specifically and reversibly to another compound, herein referred to as the "binding protein". Useful analytes and binding proteins include antigens (or haptens) and antibodies, hormones and receptors, and any other compound

combinations capable of specific binding interactions that are essentially reversible, (preferably having a dissociation constant less than about 10^{-8} M).

"Analog", as used herein, includes any compound capable of binding to the binding protein in a way analagous to analyte binding. Thus, the analog includes compounds that are the same as the analyte, as well as analogs of the analyte, such as those that have different binding affinities or that are tagged in some way (as with a fluorophore or radioisotope).

"Zero sample dose", as used herein, describes an assay run in the absence of sample analyte. The amount of free analog exiting the matrix at zero sample dose is defined as F_0.

"Free analog", as used herein, is the amount of free analog exiting the matrix unbound in a given assay. The amount of free analog exiting the matrix is defined as F.

"Dose response curve", as used herein, is the standard curve plotting the amount of free analog exiting the matrix for a range of analyte standard concentrations, for a given initial concentration of analog and binding protein, and for a given flow rate. The "fraction of free analog" (F/F_0), as used herein, is the ratio of free analog (F) to the amount of analog exiting the matrix at zero sample dose .

Matrix, as used herein, means a structure defining a surface area accessable to analyte and analog flowed through or by the surface and having binding protein immobilized thereon.

It is an object of this invention to provide a method and apparatus for the rapid, quantitative assay of an analyte that can be used as part of a protein production monitoring system for all stages of protein purification, and which can be performed under non-equilibrium conditions. Another object of the invention is to provide a method and apparatus for the sequential assay of plural, different samples of analytes. Still another object is to provide a method for the rapid assay of plural samples of an analyte to overcome the time limitations imposed by conventional immunoassay and separation techniques.

These and other objects and features of the invention will be apparent from the description, drawings, and claims that follow.

Summary of the Invention

This invention features a method for the assay of an analyte utilizing a matrix to which is attached binding protein having binding sites for an analyte, and for an analog. As disclosed herein, the method can be used to assay the quantity of analyte in a given sample by simply measuring the amount of free analog exiting the matrix. The method is rapid and quantitative and can be used to assay different analytes, or to assay plural samples of one analyte.

A method and apparatus for the rapid quantitative assay of an analyte has now been discovered. The method comprises the steps of providing a matrix having a known quantity of a binding protein capable of binding the analyte specifically attached to it The binding reaction should be essentially irreversible, preferably having a K_d of less than about 10^{-8} M. The quantity of binding protein used for a given assay is such that the binding protein occupies only a small portion of the available attachment sites on the matrix, i.e., less than 20%, and more typically less than 1.0%. A preselected volume containing the analyte is then added to the matrix. The amount of analyte in the volume should be insufficient to saturate the binding sites of the binding protein. The binding protein specifically binds the sample analyte in the volume, thereby "capturing" the analyte while all other

solutes present in the sample volume pass through the matrix. A known amount of analog is then added to the matrix. The amount added is at least equal to the total number of binding sites on the matrix. The amount of free analog (F) exiting the matrix is then quantitated and is related to the amount of analyte bound by comparison to a standard curve derived for a given flow rate, the ratio F/F_0 being directly related to the amount of analyte bound in the assay.

The assay is preferably performed under non-equilibrium conditions, allowing rapid measurement without a concomitant loss of sensitivity, and does not require quantitation of bound components, substantially eliminating the problems associated with desorption kinetics and desorption buffer interferences. Moreover, because the analog is added after the sample antigen, unbound analog will exit the matrix free of any non-analyte solutes present in the sample volume. Hence, the analog need not be tagged to be identified.

In another aspect of the invention, the assay may be run as a simultaneous assay, where both the analyte and analog are added to the matrix at the same time. In this assay, the analog must be tagged to identify it, as other solutes, including some target analyte, may coelute with free analog. The sensitivity of a simultaneous assay can be further enhanced by utilizing an analog having a lower binding affinity for the binding protein than the target analyte. In this way, more target analyte will have an opportunity to be captured as it passes

through the matrix and will be less likely to pass through unbound. This type of analog also may be used in sequential assays.

By providing a matrix having attachment sites to which the binding protein can be reversibly bound, the method of this invention may be used for the rapid, quantitative assay of plural samples of an analyte, or for the rapid, quantitative assay of different analytes. The system is easily regenerated after each assay by eluting both the binding protein and bound analyte/analog from the matrix, and reloading the matrix with fresh binding protein. A matrix surface that can be regenerated easily provides a flexible protein monitoring system readily adapted to analyze different samples. Moreover, the system need not be recalibrated between assays if all the binding protein added to the matrix in each assay will be bound by the matrix. By providing a matrix with a binding protein attachment surface large enough such that the available attachment surface is always in great excess to the amount of binding protein added for a given assay, one can assume reliably that all the binding protein added in each assay will be bound.

Thus, for the system to be useful as a rapid monitoring assay in manufacturing processes, the binding protein should be present at a fraction of the matrix's binding capacity for the binding protein in each assay. The smaller the fraction occupied in each assay, the longer the lifetime of the system. Another requirement for reliable analysis of repeated

assays is that the volume of the system remain as small as possible. Repeated use of the matrix may decrease its attachment surface capacity over time as the matrix surface breaks down. If a large volume system is used, reactions may begin to occur in isolated sub-areas or "reaction zones". The apparent binding protein concentration available within a reaction zone then may differ from the total binding protein concentration added, and the data generated may become unreliable.

For the rapid monitoring system of this invention, the requirements for a high binding protein attachment surface area and a small volume can be achieved with a chromatography system using a porous matrix, such as provided in conventional HPLC matrix material, and transporting solutes through the matrix along a pressure gradient. A 500 Å pore size, 30 μm particle size matrix material in a 0.4 ml volume column, for example, provides sufficient surface area so that each assay using 10 μg of binding protein uses only 1/25 - 1/30 of the total matrix binding capacity.

Unfortunately, the speed of conventional porous matrix HPLC analysis is limited, primarily because mass transfer within the particle pores becomes diffusive, as compared to the mass transfer between pores, which is convective. Because the intra-particle mass transfer of conventional HPLC matrices is diffusive, substantial incubation times and/or increased quantities of binding protein may be

required if all sample antigen is to "see" the binding protein. One can increase flow rates at the expense of high pressure drops, but this reduces assay sensitivity.

These limitations of conventional HPLC analysis are overcome by the use of high speed chromatographic matrices capable of perfusive chromatography. These matrices comprise particles which may be of the same overall size as are sometimes employed in conventional matrices, but having increased intraparticle porosity. In addition to intraparticle throughpores of increased diameter, e.g., 6000-8000Å, particles capable of perfusive chromatography have a network of 500-1500 Å pores interconnecting the larger throughpores. The resulting network limits the diffusional path lengths within the particles so that mass transfer within the particle pores is essentially governed by convection. The effect is to increase the mobile phase velocity of these systems to greater than 10-100 times that of conventional HPLC systems (greater than 1000cm/hr), with no substantial loss in resolution. A more detailed description of perfusive chromatography is provided in U.S. Application No. 376,885, the disclosure of which is hereby incorporated by reference. Perfusive chromatography matrix materials are available commercially from PerSeptive Biosystems, Inc., of Cambridge, Mass. U.S.A. The use of a perfusive chromatography matrix greatly enhances the speed of the assays of this invention. It is envisioned that one cycle of the

assay, from addition of binding protein to elution of all bound complexes can be performed in substantially less than 60 seconds with no significant loss of resolution.

The ability to use high flow rates without a substantial loss of resolution using a perfusive chromatography system allows the assays of this invention to be run at high speed without requiring increased quantities of binding protein. In addition to the economic savings this implies, the reduced requirement of binding protein per assay can increase the useful life of the matrix. Moreover, the increased porosity of the particles capable of perfusive chromatography substantially increases the surface area available for attachment of binding protein, typically to levels within the range of 30 to 50 m^2/ml. One ml of perfusive matrix is capable of binding approximately 10-20 mg of binding protein. Thus, the fraction of available attachment sites occupied in an assay using 10 µg of binding protein occupies less than 1/1000 of the total binding protein attachment capacity of the matrix. Put another way, for any given assay utilizing 10 µg of binding protein, the binding protein would be present at a surface density of less than 3 µg/m^2. Quantities of binding protein less than 10 µg will occupy an even smaller portion of the available attachment sites. Finally, the exceptionally high surface area provided by perfusive matrices allows one to reduce the column volume required for an assay significantly while still maintaining a large excess

of binding protein attachment surface area, essentially eliminating concerns about the occurrence of reaction zones over time.

By providing a matrix with binding protein attachment sites to which the binding protein can attach reversibly, the system can be regenerated after each assay, providing a flexible, reusable assay system. Hence, sequential samples of the same analyte or of different analytes may be measured by this method, without requiring recalibration of the system between assays. Thus, in another aspect, the invention involves a method for the rapid, quantitative sequential assay of plural samples of analytes. An assay comprises the steps of reversibly attaching a binding protein having binding sites for an analyte to a matrix such that the amount of binding protein attached to the matrix occupies a small portion of the available attachment sites on the matrix, performing a quantitative assay using the analyte binding sites on the matrix to bind the analyte from a sample volume, and then removing the binding protein from the matrix. The system is now regenerated using a second binding protein, which may have binding sites for a second, different analyte.

Another method of achieving the high surface area/low volume requirements of this invention is to use high performance capillary electrophoresis. Here the binding protein is coated onto the inner surface of a capillary tube (e.g., diameter less than 100 μm), at a surface density such that binding protein

will occupy only a fraction of the available surface for any given assay. Solutes then are transported through the capillary tube matrix along a voltage gradient created when a voltage is applied across the system. Moreover, the surface area to volume ratio of these capillary tubes is significantly greater than that of other electrophoresis systems. This high ratio provides the system with excellent heat dissipation properties, allowing one to apply larger voltages across the system, significantly reducing assay time. Thus, another aspect of the invention involves a method and apparatus for the rapid assay of analytes where the analyte is transported along a voltage gradient.

As indicated earlier, the binding protein/analyte combination can be any protein combination capable of specific reversible binding interactions. For example, one can envision the binding protein and analyte comprising any enzyme/substrate combination, including receptor and ligand, or antibody and antigen. Similarly, one can envision a number of different ways the binding protein can be reversibly bound to the matrix surface. For example, in the case where the binding protein is an immunoglobulin, the matrix surface may comprise an antibody specific to the Fc region of the binding protein antibody (such as immunoglobulins generated cross-species). Alternatively, protein A or protein G, two proteins known to bind to the Fc region of immunoglobulins may be used. Currently preferred matrices have protein A or protein G

attached to their surface. Non-specific adsorption of binding protein directly onto the surface of a chromatography matrix also may be used.

Analogs exiting the matrix may be detected in a number of different ways. In the sequential assay, free analog exits the matrix without contaminating coeluents and therefore does not need to be tagged to be identified. In the case where the analog is a protein, one can quantitate unbound analog exiting the matrix by measuring UV absorbance at 225 nm with a spectrophotometer. Alternatively, the analog may be tagged with a detectable moiety. If the reaction is run as a simultaneous assay, the analog must be tagged. A currently preferred tag is a fluorophore, although other commonly used tags, such as enzyme tags, may be useful. A fluorophore can be detected with a spectrofluorimeter, or a spectrophotometer, measuring absorbance at 495 nm. SAIAs run with fluorescently tagged analogs also provide an internal "self check" mechanism to verify that other proteins are not coeluting with the analog. Only the analog will be detected at 495 nm, but all proteins present in the eluent will absorb at 225 nm.

Finally, the apparatus of the invention may provide a means of calculating and displaying the amount of analyte present in the analyzed sample.

Brief Description of the Drawings

Figures 1A and 1B are schematic diagrams illustrating methods of the invention;

Figure 2 is a schematic representation of an apparatus embodying the invention; and

Figures 3, 4, 5 and 6 are graphs illustrating various principles and characteristics of the present invention.

Like referenced characters in respective drawn figures indicate corresponding parts.

Detailed Description

In its broadest sense, this invention describes a method and apparatus for the rapid assay of an analyte. The process is particularly useful as a monitor of protein quality in a protein production process.

The method of the invention may be understood by referring to the schematic diagram of a sequential assay, depicted in Figure 1A. A matrix 10 is provided having on its surface attachment sites 12 capable of specific and reversible interaction with a binding protein 14 having binding sites 16 for the analyte to be measured. The quantity of binding protein 14 added to the matrix is a small fraction of the attachment sites 12 available for attaching the binding protein to the matrix. This ensures that all the binding protein added interacts with attachment sites on the matrix to form a complex 18. A volume containing the analyte 20 to be sampled, and optionally, other non-analyte solute components 22, is then flowed through the matrix system. The amount of the analyte added must be insufficient to saturate the binding protein binding sites 16. The analyte 20 interacts specifically with these binding sites 16 to form a complex 24, while non-specific components 22 pass through the matrix unbound. Next, an analog 26, identical or analogous to the sample analyte, is added to the matrix system in an amount at least equivalent to the number of the binding sites 16 on the matrix. The analog, capable of specifically binding with the binding protein 14, forms complexes

24 with remaining binding sites 16 on the binding protein 14. Free analog passes through the matrix unbound and is directed by a detector 28 which monitors the matrix effluent.

The sequential assay of this invention is most useful when performed under nonequilibrium conditions. Provided flow rates are uniform in calibration runs and analysis runs, there is no requirement that the binding reactions reach equilibrium. As such, the amount of analyte-binding protein complex formed for any given assay is dependent on the concentration of the reactants and the length of time that the reaction is allowed to progress. Accordingly, the system provides the experimenter with a broad experimental range, as the experimenter can vary both the concentration of the reactants and the duration of the experiment, as desired.

Reproducibility of assays using the system depends on providing a matrix having a sufficient excess of attachment sites for the binding protein such that all the binding protein added for a given assay will bind to the matrix. A calibration curve can be determined by preparing a standard dose response curve using samples having a range of analyte concentration, using a given binding protein matrix, a given analog concentration, and a given flow rate. The standard curve can be determined experimentally or, where the rate constants for the reaction are known, by calculation. The amount of analog exiting the matrix at zero sample dose (F_0) can be m asured experimentally, or extrapolated

from the standard curve. An additional advantage of the present system is that, provided the reaction time (flow rate) is sufficient to allow all sample analyte to bind, the choice of flow rate is not critical, as the amount of free analog exiting the matrix will always be a function of the amount of analyte bound. While a slower flow rate (longer reaction time) allows a greater fraction of analog to bind, it is not required for quantitation.

An analogous system is depicted in Figure 1B, using a simultaneous introduction and flow of sample and analog through the column. Here, analog 26 must be tagged to differentiate it from sample analyte 20 and other, non-specific compounds 22. Preferably, the analog has a lower binding affinity for the binding protein than the analyte, thereby allowing more analyte to bind, and providing a more sensitive assay.

Sequential assays made on a product stream from a protein production system or on other types of protein solutions can be carried out using an apparatus such as that depicted schematically in Figure 2. The apparatus preferably comprises a multi-port sampling valve 28 such as is found in automated protein production systems known to those skilled in the art, and which allocates the various components of the assay to matrix 40, whose surface is capable of reversibly binding the binding protein. Thus, reservoirs containing buffer 30, binding protein 32, analog 36, and a recycling

solvent 38, and means for obtaining a sample 34, e.g., an accumulator, controlled by valve 28, provide fluids to matrix 40, impelled by, e.g., pump 31. The recycling solvent 38 is capable of desorbing all reversibly bound complexes from the matrix surface.

Valve position of the multi-port sampling valve 28 is preferably under computer control. In addition, buffer delivery to the matrix may be driven by a metering pump 29. Effluent exiting the matrix is either discarded through line 42, or quantitated in a detector, 44, e.g., a conventional detector which measures U.V. absorbence through a film of fluid, and produces a curve which, when integrated, gives quantitative information. Optionally, the apparatus may further comprise a means 46 for calculating analyte concentration and for displaying data indicative of the amount of analyte in the sample.

In operation, the column 40 is maintained either at equilibrium with buffer from reservoir 30, or preferably in a rest state in which it is loaded with binding protein from reservoir 32. To initiate an assay, the system is activated to cycle through a series of steps involving flowing at a predetermined flow rate a metered quantity of sample 34, followed by a metered quantity of analog 36. Alternatively, if analog 36 is distinguished from the sample by the detector 44 (e.g., if the analog is tagged), the sample and analog may be passed through matrix 40 together. Effluent from the column produced by the

breakthrough of sample 36 is measured for protein concentration by detector 44. The amount of analog detected is then correlated to analyte concentration by, for example, electronic comparator means in module 46.

Examples

The following examples further describe the general method of the invention. All assays were performed using conventional HPLC matrix packing materials.

Human transferrin (HTr) was the analyte measured in the protein monitoring system of the present examples. Serum samples containing HTr were obtained from St. Elizabeth's Hospital, Lafayette, IL. Pure HTr was purchased from Sigma Corp., St. Louis, MO. Rabbit anti-HTr (AHTr) was used as the binding protein, (Boehringer Mannheim Biochemicals, Indianapolis, IN), obtained as an IgG fraction of antiserum. A conventional protein A column was used as the reversible matrix binding surface (Chromatochem, Inc., Missoula, MT). Protein G columns also were used to verify several principles of the invention.

All examples were performed using a protein A (or protein G) affinity chromatography matrix (500Å, 30 μm), in a 3 x 0.2 cm i.d. stainless steel column. A Hewlett-Packard analytical workstation was used as an automated system, with a ternary gradient

liquid chromatograph (Model 1090M), together with an autosampler, autoinjector, diode array detector, and a computer system for control and integration. Detection was at 225 nm for all assays using unlabeled HTr and at 495 nm for assays using FITC-labelled HTr.

FITC labeling of human transferrin (HTr)

HTr was labeled with fluorescein isothiocyanate, Isomer I (FITC, 10%, on Cellite) using the method of Rinderknect (Nature 193:167 (1962)) for the simultaneous incubation studies. Briefly, HTr was dissolved in 0.05 M sodium carbonate (pH 8.5). To this solution was added approximat ly 15 mg of Cellite, containing 10% FITC (Sigma Co., St. Louis, MO). The mixture was shaken for 4 minutes, and then centrifuged for 3 minutes. This treatment was sufficient to transfer label to the protein, and the FITC-labeled protein was purified by column chromatography (Sephadex G25, in 0.01 M phosphate, 0.15 M NaCl, pH 7.0 buffer). The FITC to protein ratio (F/P) was determined from the concentration of protein and FITC obtained from absorbance at 280nm and absorbance at 495nm (The, et. al., Immunology 18:865 (1970)).

Evaluation of matrix column

The binding protein (AHTr) capacity of the protein A affinity chromatography matrix was evaluated by frontal analysis (Jacobson et al., J. Chromatogr. 316:53 (1984)). Duplicate 3.5 ml samples

of a 0.25 mg/ml solution of AHTr in a buffer containing 0.01 M sodium phosphate, 0.15 M NaCl (pH 7.0) was loaded onto the affinity column at 0.5 ml/min until a breakthrough curve appeared. AHTr was desorbed with 0.1 M glycine, 2% acetic acid (pH 2.9).

The binding protein capacity of this column was determined to be 0.51 mg AHTr. Thus, a given assay using 10-20 µg will only use about 1/25th of the column's capacity.

Nonspecific binding of HTr to protein A or protein G columns was measured by successive injections of HTr (1 µl, 2 mg/ml). Five successive injections yielded constant peaks for each injection, indicating that nonspecific binding of HTr to the affinity column was very low. Nonspecific binding would have resulted in peak areas slowly increasing with each injection until a maximum was reached.

The ability of analog protein to displace sample antigen from the column was tested using fluorescent labeled HTr (FITC-labeled HTr), monitoring protein at both 225 nm and 495 nm and plotting the fraction of unbound analog, F/F_o vs dose (amount of analyte in sample). As shown in Figure 3, graphs of the response curves at both wavelengths were essentially the same, indicating that no unlabeled antigen was displaced by the analog. If displacement had occurred, the F/F_o values at 225 nm would be greater than those at 495 nm.

Method for sequential addition immunoassays (SAIA)

Loading buffer for all analyses was 0.01 M sodium phosphate, 0.15 M NaCl (pH 7.0). AHTr (2 µl) was injected into the protein A column at the same flow rate (2 ml/min.) for all analyses. This amount of AHTr will theoretically bind up to 4 µg of HTr, calculated from the manufacturer's reported titer (2 µg HTr/µl AHTr). The flow rate was then adjusted to 1 ml/min and either 1 µl injections of HTr standards or aliquots of 1 µl of human serum were injected into the affinity column.

Next, tagged analog (pure HTr, 1 µl, at 4 µg/µl) was injected and the peak area of the free analog exiting the matrix was measured. Finally, antigen-antibody complexes were desorbed from the column at 2 ml/min., using 2 ml of desorbing agent. The column was then re-equilibrated.

Method for simultaneous addition of immunoassays

The method was essentially the same as for SAIAs except that antigen standards (or human serum) and FITC-labeled HTr (1 µl each) were mixed in the needle of the autoinjector prior to injection into the protein A column.

The chromatogram of a typical SAIA is shown in Figure 4. Eluents were measured by UV absorbance at 225 nm and the free fraction of analog quantitated by peak area. The peak area labelled A comprises nonspecific eluents from the antiserum IgG fraction. The peak labelled B indicates eluted

nonspecific components from the serum sample. Peak C contains unbound analog eluting from the matrix. Finally, peak D contains all bound complexes desorbed from the matrix surface.

A number of desorbing agents were tested for their ability to remove antibody-antigen complexes from the protein A columns. Protein A and immunoglobulins have two types of interactions: hydrophobic and ionic. The most effective desorbing agent found so far has proven to be a 50% ethylene glycol/10% acetic acid solution, as the ethylene glycol helps to dissociate the hydrophobic interaction, and the acetic acid affects the ionic interaction. Twenty percent acetic acid has also been used, as have 0.1 M glycine and 2% acetic acid solutions.

The absorbance spectrum of a typical, simultaneous incubation immunoassay using FITC-HTr is shown in Figure 5. The free fraction of analog was quantified by peak area measured at 495 nm. The 280 nm peak measured the absorbance of all unbound protein components, including analog, eluting from the matrix.

Figure 6 compares theoretical and experimentally derived dose response curves for four different flow rates: (A.) 2.0 ml/min; (B.) 1.0 ml/min; (C.) 0.5 ml/min; and (D.) 0.1 ml/min. As illustrated, in all cases the curves corresponded remarkably well. The theoretical curves were derived using numerical solutions for the rate equations as

described in Rodbard t al., (1971, J. Clin. Endocrinol., 33:343-355). Binding protein volume was assumed to be the void volume of the column, and sample and analyte volumes were calculated from the peakwidth at half height on chromatograms. Time was described as the ratio of the column void volume to flow rate. The experimental curves were determined by conducting multiple assays using the procedure disclosed herein.

For the present system, flow rates of 1 ml/min were found to be optimal. Optimization of the assay using conventional HPLC matrices can decrease the time required to run each cycle to approximately five minutes. Use of a perfusive matrix can increase the throughput speed of the assay substantially. In addition, use of a perfusive matrix, with its exceptionally high surface area, can also increase the dynamic range of the assay.

The invention may be embodied in other specific forms without departing from the spirit and the central characteristics thereof. Accordingly, other embodiments are within the following claims.

What is claimed is:

1. A method for the rapid, quantitative assay of an analyte, the method comprising the steps of:

A. attaching a known quantity of a binding protein having analyte binding sites to a matrix having attachment sites for said binding protein such that said binding protein occupies only a small portion of the available attachment sites on the matrix;

B. loading the matrix of step A with a preselected volume of sample containing an amount of the analyte insufficient to saturate the binding sites of said binding protein;

C. flowing a known amount of an analog through said matrix, said amount being at least sufficient to saturate the binding sites on the matrix; and

D. measuring the amount of free analog exiting said matrix, the amount being directly related to the amount of analyte in the sample.

2. A method useful for sequential rapid assay of plural samples containing an analyte, the method comprising the steps of:

A. reversibly attaching a known quantity of a binding protein having analyte binding sites to attachment sites on a matrix for said binding protein, said quantity being such that said binding protein occupies only a small portion of the available attachment sites on said matrix;

B. loading the matrix of step A with a preselected volume of sample, containing an amount of the analyte insufficient to saturate the binding sites of said binding protein;

C. flowing a known amount of an analog through said matrix, said amount being at least sufficient to saturate the binding sites on the matrix;

D. measuring the amount of free analog exiting said matrix, the amount being related to the quantity of analyte in the sample;

E. removing the binding protein from said matrix; and

F. repeating steps A through E with a different sample.

3. A method for rapid, sequential, quantitative assay of plural samples containing an analyte, the method comprising the steps of:

A. reversibly attaching a binding protein having analyte binding sites to attachment sites on a matrix for said binding protein, such that said binding protein occupies only a small portion of the available attachment sites on said matrix;

B. performing a quantitative assay using the analyte binding sites on the matrix of step A to bind analyte from a first said sample;

C. removing the binding protein from said matrix; and

D. repeating steps A, B, and C using a second said sample.

4. The method of claim 1, 2 or 3 wherein said assay is performed under nonequilibrium conditions.

5. The method of claim 1, 2 or 3 wherein said matrix attachment sites comprise protein A or protein G, and said binding protein comprises an immunoglobulin which binds to said analyte.

6. The method of claim 1, 2 or 3 wherein said analog has a lower binding affinity for said binding protein than said analyte.

7. The method of claim 1,2 or 3 wherein said analog is tagged with a detectable moiety.

8. The method of claim 7 wherein said analog is tagged with a fluorophore.

9. The method of claim 1, 2 or 3 wherein sample is transported through said matrix along a voltage gradient.

10. The method of claim 1, 2, or 3 wherein sample is transported through said matrix along a pressure gradient.

11. An apparatus for sequential rapid assay of plural samples containing an analyte, said apparatus comprising:

a matrix defining a surface area comprising means for reversibly immobilizing a binding protein having binding sites for said analyte;

means for contacting said matrix surface area with said binding protein to immobilize all of said binding protein thereon, the quantity of immobilized binding protein occupying a small fraction of the available matrix surface area;

means for contacting binding protein immobilized on the surface of said matrix with a sample;

means for detecting the presence or amount in a solution exiting said matrix of a member selected from the group consisting of said analyte, an analog of said analyte, said analyte tagged with a detectable moiety, or an analog of said analyte tagged with a detectable moiety; and

means for removing said binding protein from said matrix.

11. The apparatus of claim 11 wherein said means for detecting is a ultra-violet spectrophotometer.

12. The apparatus of claim 11 wherein said means for detecting is a spectrofluorimeter.

13. The apparatus of claim 11 wherein said means for reversibly immobilizing binding protein is a surface coating of protein A or protein G on said matrix.

14. The apparatus of claim 11 further comprising means for displaying data indicative of the amount of analyte in an analyzed sample.

15. The apparatus of claim 11 further comprising means for cycling said apparatus through an analysis and means for triggering an analysis cycle.

16. The apparatus of claim 11 wherein said matrix comprises a chromatography matrix, the apparatus further comprising means for pumping the solutions through said chromatography matrix.

17. The apparatus of claim 11 wherein said matrix comprises an electrophoresis apparatus including means for maintaining a voltage gradient across said matrix.

Fig. 1A

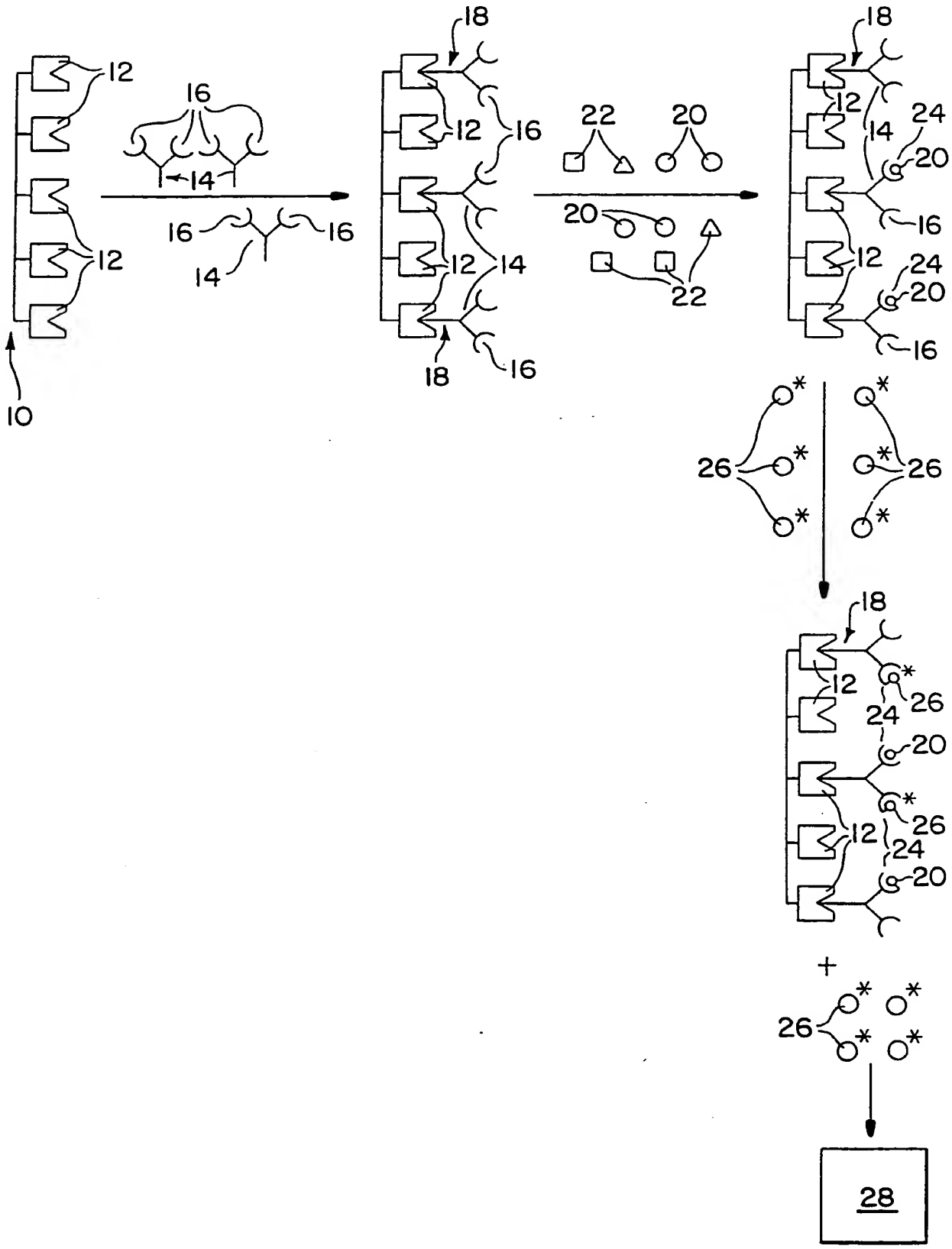


Fig. 1B

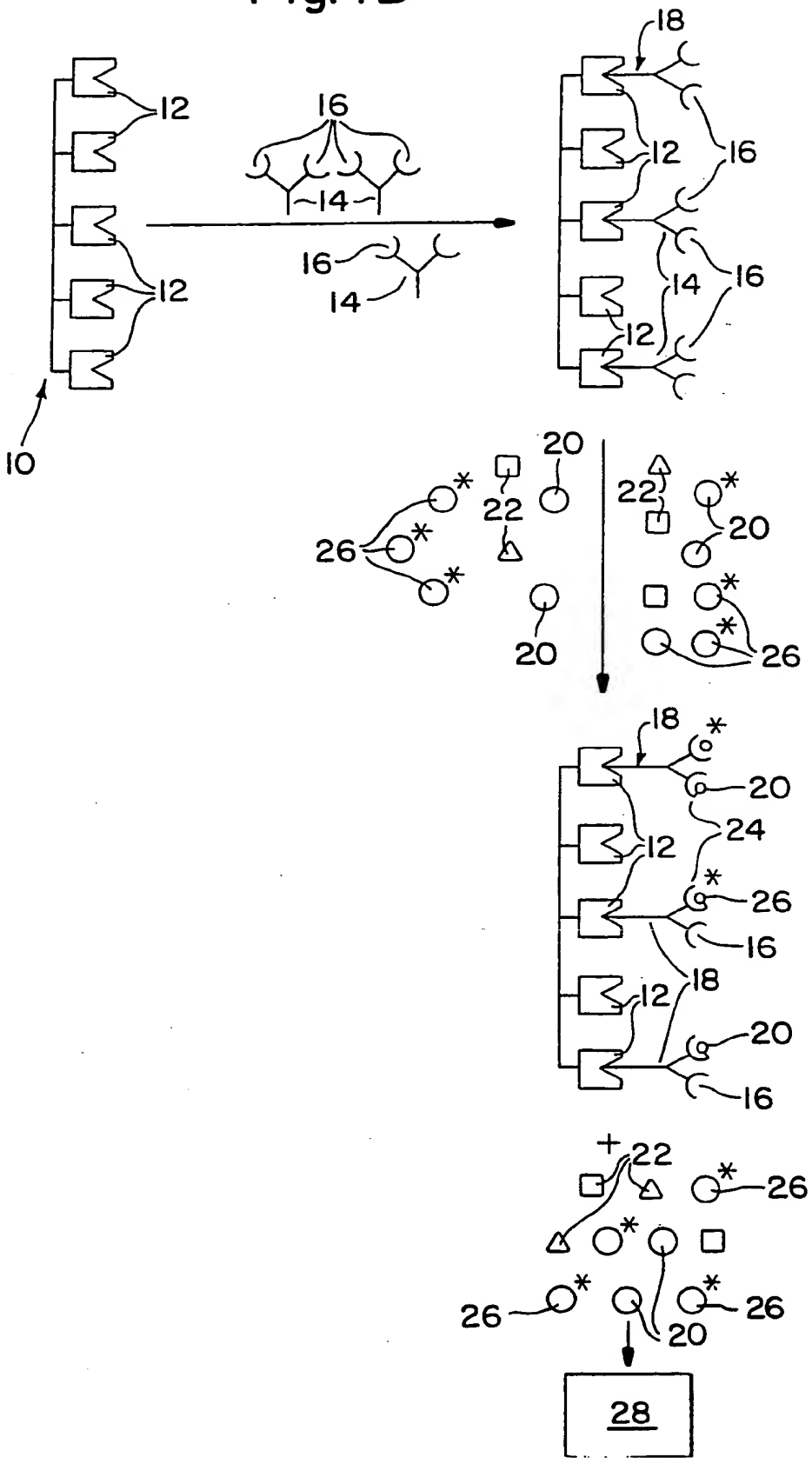


Fig. 2

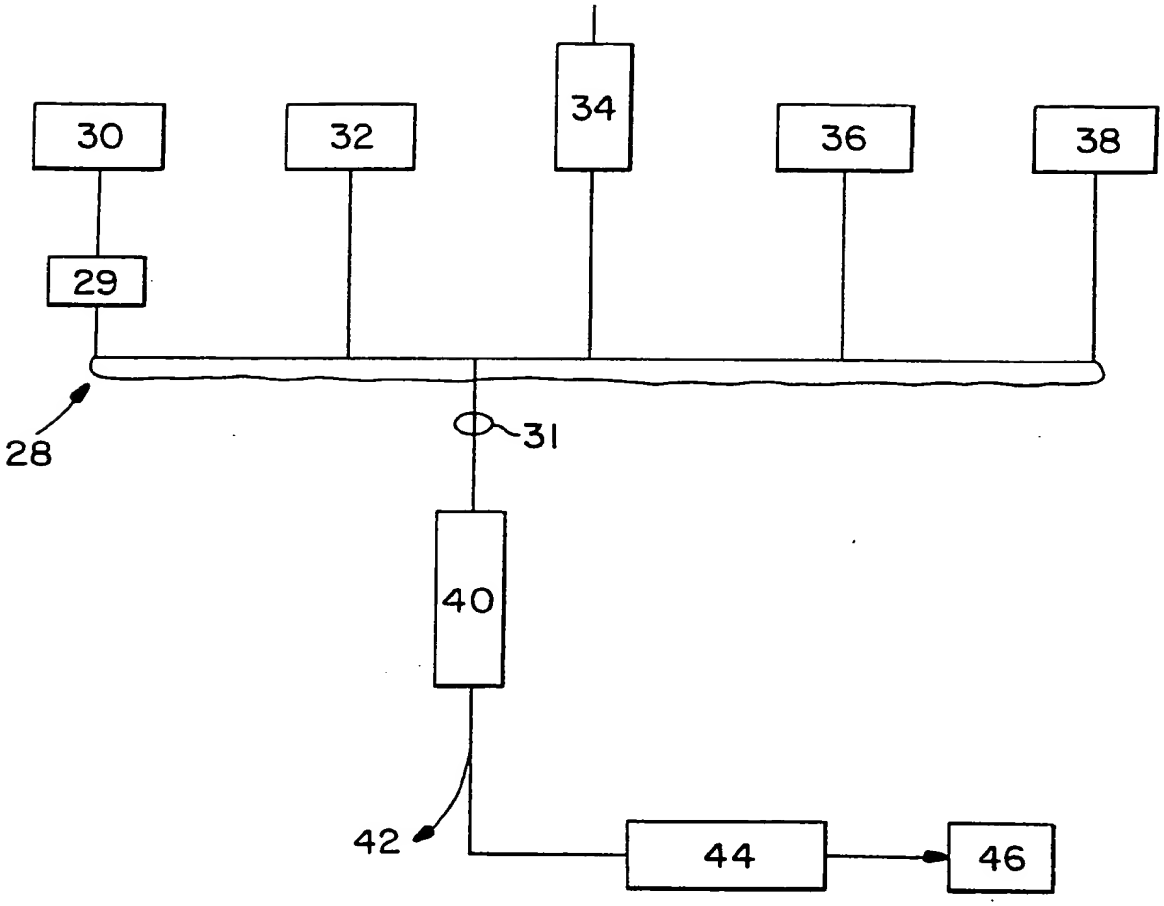


Fig. 3

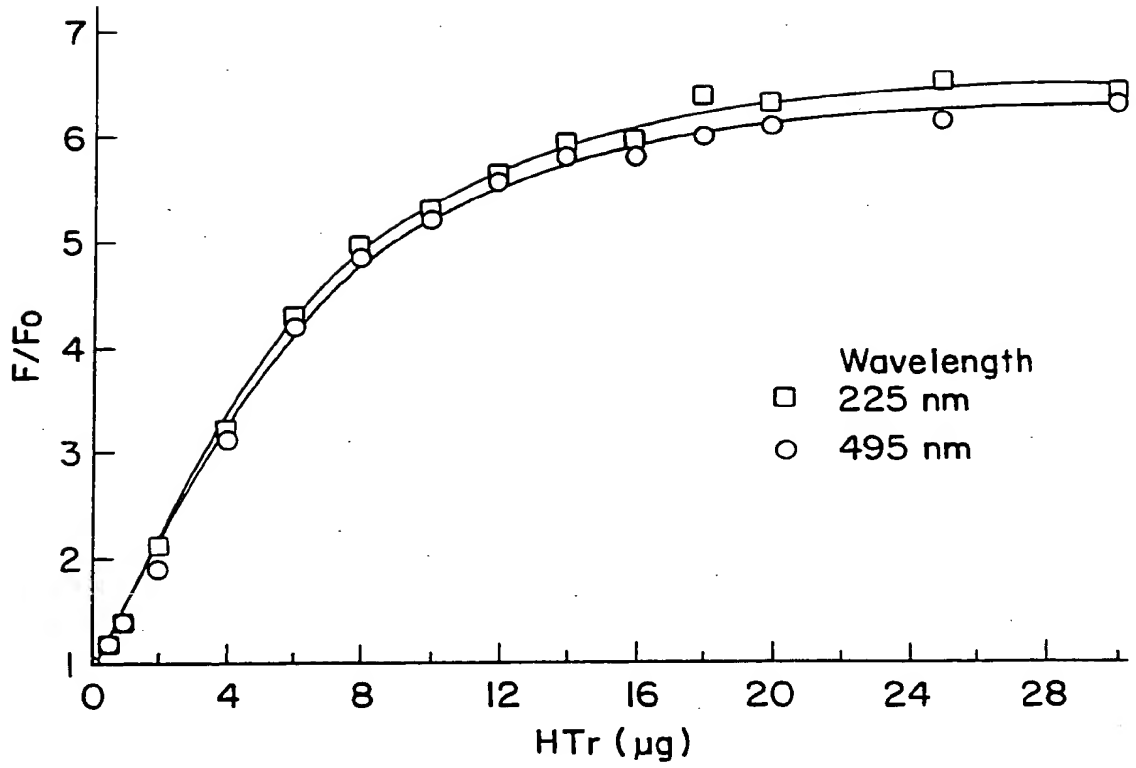


Fig. 4

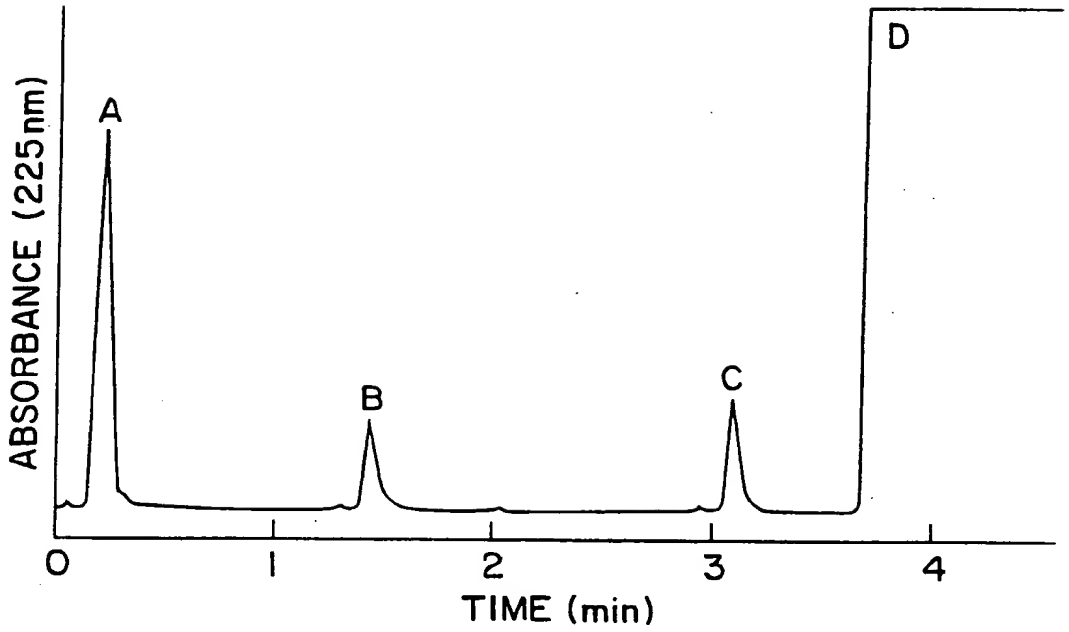


Fig. 5

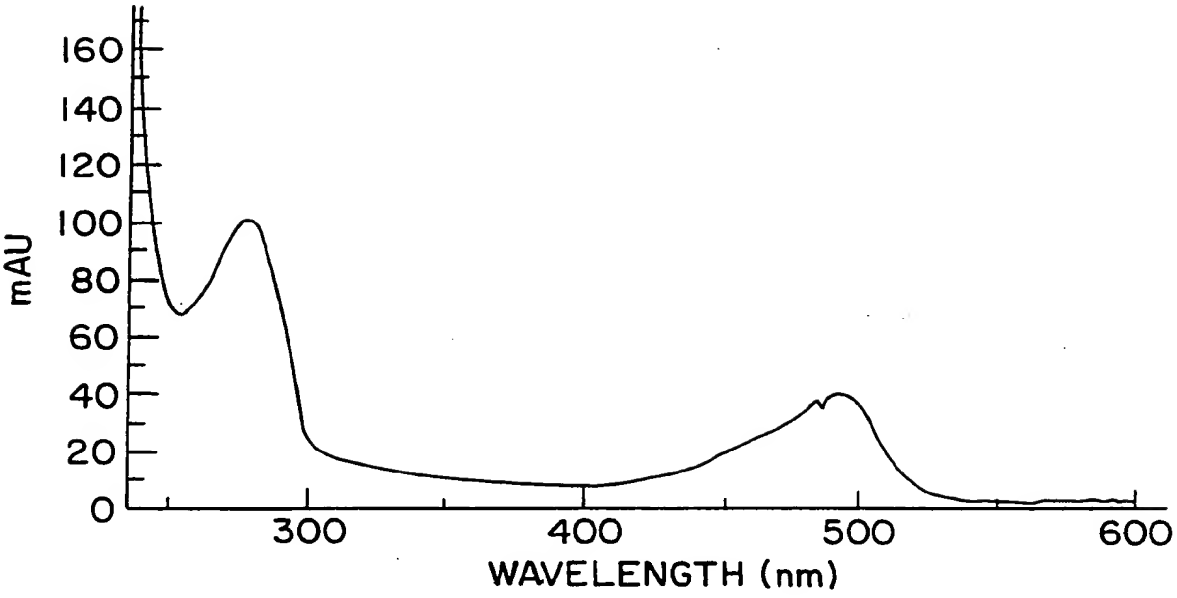


Fig. 6A

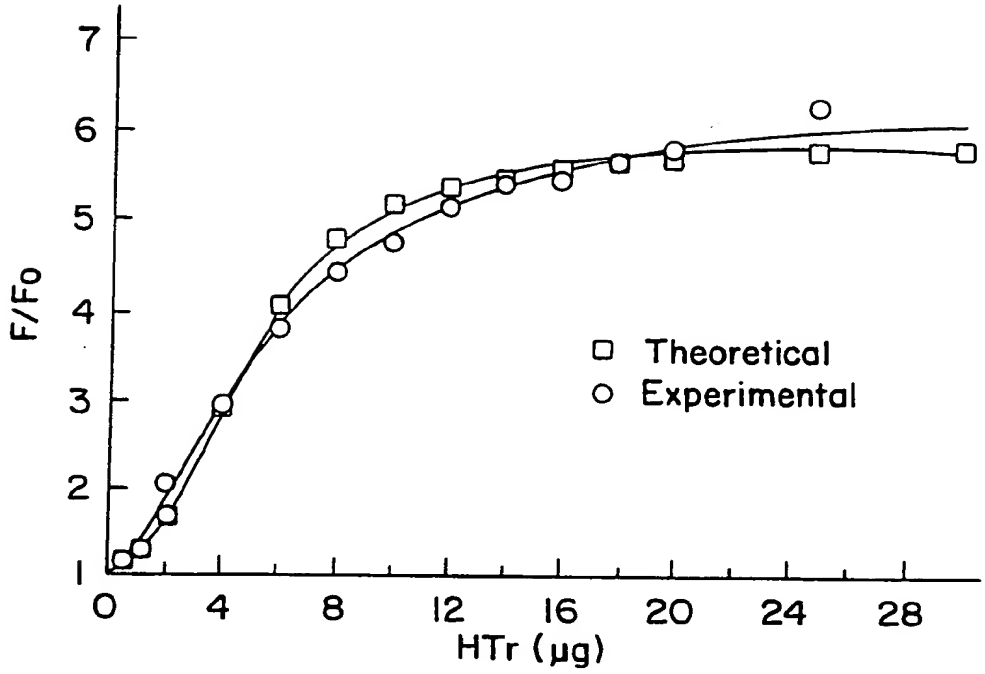

F/Fo
HTr (µg)
Theoretical
Experimental

Fig. 6B

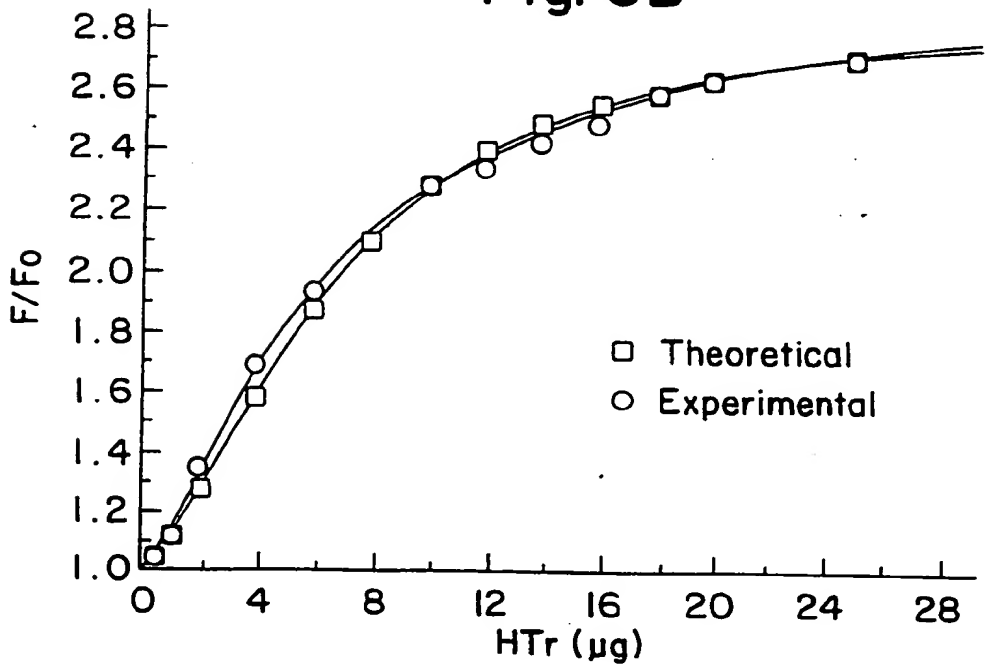

F/Fo
HTr (µg)
Theoretical
Experimental

Fig. 6C

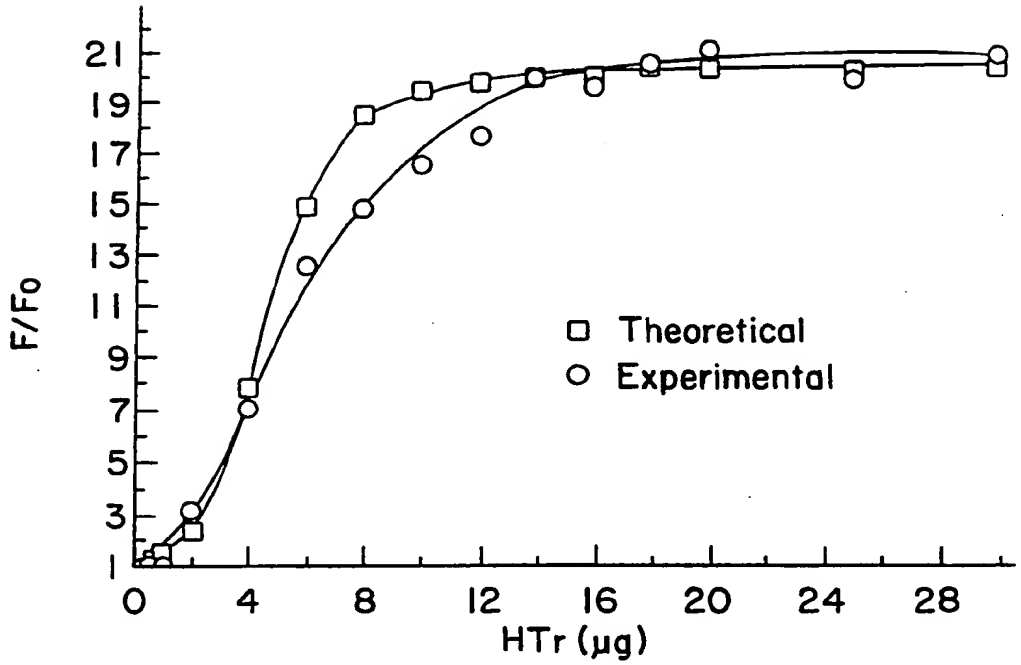
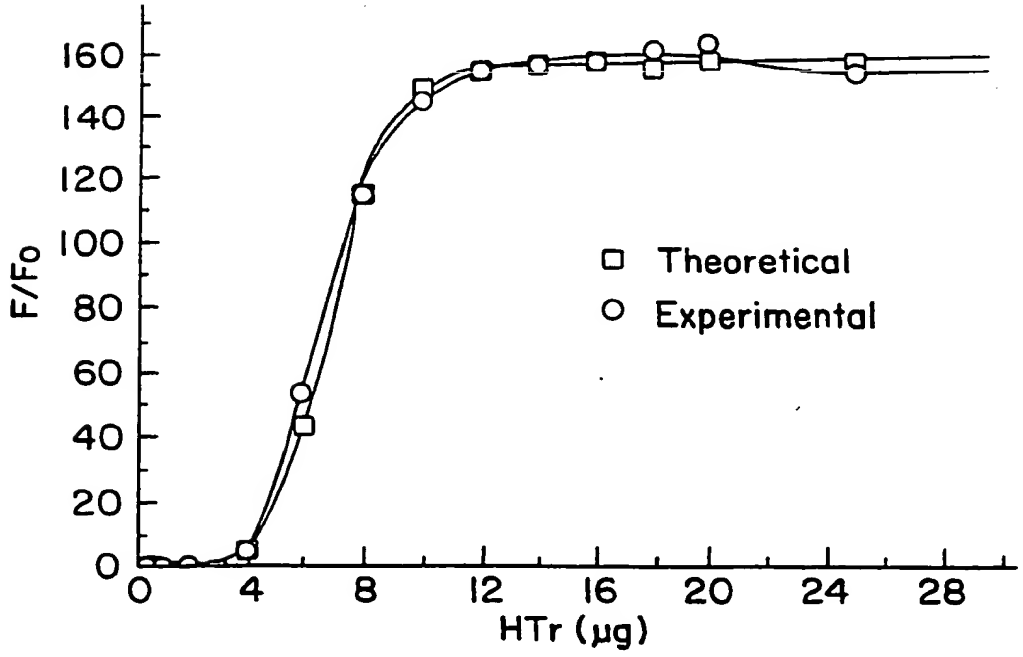

F/Fo
21
19
17
15
13
11
9
7
5
3
1
0
4
8
12
16
20
24
28
HTr (µg)
Theoretical
Experimental

Fig. 6D


F/Fo
160
140
120
100
80
60
40
20
0
0
4
8
12
16
20
24
28
HTr (µg)
Theoretical
Experimental

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 91/05694

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 G01N33/543; G01N33/561

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	G01N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X Y	WO,A,8 606 170 (IMMUNICON CORP.) 23 October 1986 see page 3, line 29 - page 4, line 9 see page 14, line 22 - page 20, line 13; claims	1,4 2,3
X	DE,A,3 617 946 (HITACHI, LTD.) 4 December 1986 see the whole document esp. page 3, line 28-page 6, line 6	1,9,11, 17
Y	DE,A,3 235 516 (AMANO PHARMACEUTICAL CO., LTD.) 21 April 1983 see page 6, line 29 - page 14, line 3	2,3
A	FR,A,2 247 728 (MILES LABORATORIES, INC.) 9 May 1975 see page 4, line 1 - page 8, line 10 see page 13, line 25 - page 15, line 7	1-4

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° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
19 DECEMBER 1991	14. 01. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	LUZZATTO E.R.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
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A	US,A,4 801 726 (R.W.GIESE ET AL.) 31 January 1989 see column 6, line 61 - column 7, line 23; figure 3	1
A	US,A,4 434 236 (J.W.FREYTAG) 28 February 1984 see the whole document esp. abstract	6
A	AT,A,371 606 (CHANDON INVESTMENT PLANNING LTD.) 11 July 1983 see the whole document esp. page 2, lines42-48, page 4, lines17-51	1
A	WO,A,8 808 978 (THE MCLEAN HOSPITAL CORPORATION) 17 November 1988 see the whole document esp. page 6, line3-page 7, line25	1
A	P.TIJSSEN 'Practice and theory of enzyme immunoassays' 1985 , ELSEVIER , AMSTERDAM see page 142, line 9 - page 143, line 26	1-4
A	US,A,4 895 809 (T.D.SCHLABACH ET AL.) 23 January 1990 see the whole document	3

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9105694 SA 51846

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on
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US-A-4895809	23-01-90	None		

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